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AMYLOSE CHANGES IN THE STARCH OF DEVELOPING WHEAT GRAINS

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Abstract—Amylose fractions, obtained from the starches of young grains of wheat by the usual method of complexing with *n*-butanol, bound small quantities of iodine compared with amylose isolated from mature tissues, suggesting a structural difference to the essentially unbranched α(1–4)glucan chain of the latter. However, fractionation by preparative ultra-centrifugation or by exclusion chromatography on agarose gel showed that these low binding values of amyloses isolated by complexing procedures from immature wheat were associated with incomplete separation from amylopectin and that the increase in the iodine binding of starch that accompanies its accumulation in developing wheat grains is due to an increase in the proportion of unbranched α(1–4)glucan.

INTRODUCTION

THE STARCHES from a range of developing plant organs show an increase in the isopotential absorption of iodine as they accumulate. Wheat[1, 2] and barley grains,[3] maize[4] and pea seeds,[5] potato tubers[6] and tobacco leaves[7] all have this pattern. The isopotential binding of iodine by the whole starches has been used in most of these examples to estimate the apparent amylose content. In these calculations it has been assumed that the binding of the amylose remains constant throughout the accumulation of starch. The results indicate increasing proportions of amylose to amylopectin during development. Usually a value of about 19 g of iodine per 100 g of amylose is used and this is derived from the binding of amylose from mature potato tuber granules, prepared by the currently available method of complexing with compounds such as *n*-butanol.[8] Mature potato amylose is an essentially unbranched α(1–4)glucan that may have very occasional branch points.[9, 10]

However, it is possible that the iodine binding of the amylose changes during growth. For true values of the amylose content, each sample should be completely fractionated and the iodine binding of the separated amylose determined and used for calculation. In one instance where this has been done,[2] the amylose from wheat grains at 4 and 6 weeks after flowering bound 14·4 and 17·0 g of iodine. The value for mature grains was 21. One consequence of these results is that the amylose content of wheat may be calculated to change only slightly during development, as the binding values of the whole starches were 3·0,

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3·8 and 4·2. Structural changes occurring in the amylose during accumulation may account for these results, as the amount of iodine bound is a function of the length of uninterrupted α(1–4) linked D-glucose units.[11] Possible structural changes that could affect binding are the introduction of a number of α(1–6) branch points, the presence of larger amounts of amylose of a much lower degree of polymerisation (about 100) in the amylose component or of occasional glycosidic linkages other than α(1–4). Structural changes of this type could be relevant to the biosynthesis of starch, as the diurnal accumulation and nocturnal depletion of starch in tobacco leaves, in the expansion phase of growth, is also accompanied by changes in the isopotential binding of the whole starches.[12]

However, another possible reason for the lower iodine binding is that immature and mature starches behave differently on fractionation with *n*-butanol or similar compounds. There is evidence for the presence of more than two fractions in mature maize[13] and wheat starches[14] and for an amylose fraction containing amylopectin from mature wrinkled-seeded peas that contain a high amylose starch.[15]

The aim of this work has been to find if the increase in binding of whole starches in developing wheat grains is due to alteration in the structure of the amylose, causing changes in the binding of iodine per unit weight of amylose, or to an increase in the amount of essentially unbranched amylose.

RESULTS AND DISCUSSION

Wheat spikes were marked at anthesis. The grain collected 14 and 42 days later had a dry weight of 7 and 35 mg per seed. The water contents were 72% and 28% and the starch contents, on a dry weight basis, were 18% and 56%. The anhydro glucose content of the 14 day granules was 86% and of the 42 day granules was 88%. These values are typical for isolated starch granules and most of the remainder is firmly bound water. The younger granules contained 0·013% of phosphate and 0·012% of N and the older 0·043% of phosphate and 0·021% of N on a dry weight basis.

The isopotential binding values of these two samples (Table 1) were lower than those of

TABLE 1. ISOPOTENTIAL IODINE BINDING AND APPARENT AMYLOSE CONTENTS OF STARCHES

Sample	Iodine binding (g iodine/100 g of sample)	Apparent amylose content* (%)
Wheat starch 2 weeks after anthesis	2·7	14·0
Wheat starch 6 weeks after anthesis	3·8	19·8
Potato starch	4·6	23·8

* Calculated by assuming that amylose binds 19·2 g iodine per 100 g.

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mature starches and showed the expected increase as the grain developed. A sample from mature potato tubers showed values similar to those published.

The starches were dispersed in dimethyl sulphoxide under standardised conditions. It was essential to use peroxide-free ether in the washing and the dissolution of the starches in aqueous solution, otherwise extensive depolymerisation occurred. The conditions of centrifuging of the *n*-butanol complexes were also standard and gave no precipitate before the addition of *n*-butanol. In agreement with previous results,[2] in a number of fractionations under optimal conditions[8] which involved complexing twice, the amylose from immature wheat starches showed binding values lower than those found for mature starches. These were in the range 12–13 g of iodine per 100 g of fraction from 14 day starch and in the range 14–17, but mostly 16–17, for 42 day starch. The addition of thymol in place of *n*-butanol still gave similar low values indicating that the effect is not only associated with complexing with *n*-butanol. Re-dissolution in dilute NaCl solution and complexing again gave only slight increases in the iodine binding. Potato amylose showed values similar to previously published figures (18·5 g iodine per 100 g amylose). The actual amount of binding found varies slightly with the conditions of titration. Fractionation of the starches from rapidly expanding tobacco leaves and from immature, smooth-seeded peas also gave iodine binding values lower than those found for the amyloses of mature tissues, suggesting that the effect is associated generally with immature starches. However, because of the possibility that the fractionation procedure functions differently in mature and inmature granules, alternative methods of separation were studied.

The wheat and potato starches were fractionated by preparative ultra-centrifugation at 65,000 rev/min in a swinging bucket rotor, using band forming caps, with a sucrose gradient to stabilize the separated compounds. Two components were indicated from the

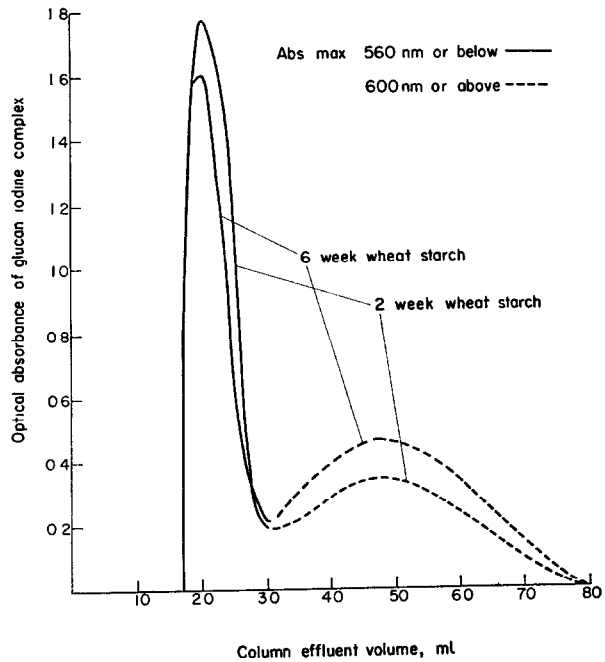

FIG. 1. CHROMATOGRAPHY ON 2% AGAROSE GEL OF WHEAT STARCHES.

glucan-iodine colours of the fractions obtained on piercing the tubes. The heavier component, which had an absorption maximum at 560 nm, corresponded to amylopectin. The absorption values were very high at the base of the tube and decreased to low values at the 2·0 ml level. The lighter component (3·3 ml level) had an absorption maximum at 600 nm or above, indicating that it was amylose. The absorption values of the fractions of this component for the three starch samples were plotted and the area under the curves was approximately proportional to the isopotential binding of the whole starches. The two wheat starches were also fractionated by exclusion chromatography on 2% agarose gel with an exclusion limit for polysaccharides of 20×10^6. Measurement of the glucan-iodine absorption spectra of the eluate fractions gave curves with two peaks, as shown in Fig. 1. Fractions of the excluded component had absorption maxima at 560 nm and of the retarded component at 600 nm or above. From the known molecular weights and iodine complex absorption spectra, the excluded component was amylopectin and the retarded amylose. Two week starch had a larger amylopectin component and a smaller amount of amylose component than 6 week starch. Assuming that each of the more slowly sedimenting components from ultra-centrifuging of the three starches and each of the retarded components from chromatography of the two wheat starches had the same spectral absorptions of the glucan-iodine complexes per unit weight of glucan, then these results would show that the differences in the isopotential binding of the whole starches reflect differences in the amounts of essentially unbranched amylose. This is in contrast to the results of *n*-butanol complexing. However, the different areas under the curves could be due to structural differences in these components that produce different absorption spectra.

Agarose gel chromatography of the amylopectins from *n*-butanol fractionation gave the elution patterns shown in Fig. 2. The area due to amylopectin is smaller than in the whole starches (Fig. 1) and there has been a greater decrease in the 2 week than the 6 week sample. Chromatography of the mother liquor fractions is also depicted in Fig. 2. The

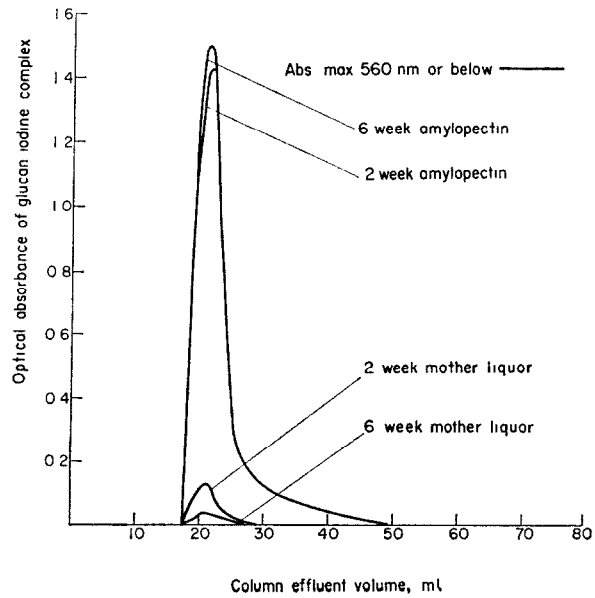

FIG. 2. CHROMATOGRAPHY ON 2% AGAROSE GEL OF WHEAT STARCH FRACTIONS.

molecular size as indicated by chromatography and the wavelength of absorption maxima of the iodine complexes, show that these fractions were amylopectin. The amount of this fraction was dependent on the force, time and temperature of centrifugation of the amylose–butanol complex, making it necessary to adopt standard conditions for all fractionations. The results suggest that the amyloses of the 2 week starch obtained by *n*-butanol fractionation and, to a lesser extent, of the 6 week starch were mixed with amylopectin.

Since differences in the areas of the curves plotted for the amylose fractions could be due to differences in the iodine absorption spectra per unit weight of glucan, the α(1–4) (1–6) glucan contents of the retarded fractions were estimated from the amount of glucose released by amyloglucosidase. The concentration of glucan was then plotted against the light absorption of the glucan-iodine complex at the wavelength of the maximum value. This gave a linear plot for the potato and the two wheat samples that could be extended to the origin, indicating that light absorption was proportional to concentration. Thus the area differences of the amylose fractions found with ultracentrifugation and with agarose gel exclusion chromatography (Fig. 1) are due to differences in the amounts of amylose and not to differences in the absorption spectra.

Using preparative columns of agarose and chromatographing repeatedly, the amylose from one gram of the 2 week starch was separated. The large volume of eluate was concentrated by membrane ultra-filtration under pressure, using a membrane with a cut-off point at a molecular weight of 10,000. This amylose fraction was found to have an isopotential iodine binding of 19·0 showing that the retarded fraction is an essentially linear amylose with an iodine binding similar to that of mature starch.

Finally, evidence was obtained that there was amylopectin in the amyloses prepared by *n*-butanol complexing. These were dispersed in dimethyl sulphoxide in the same way as

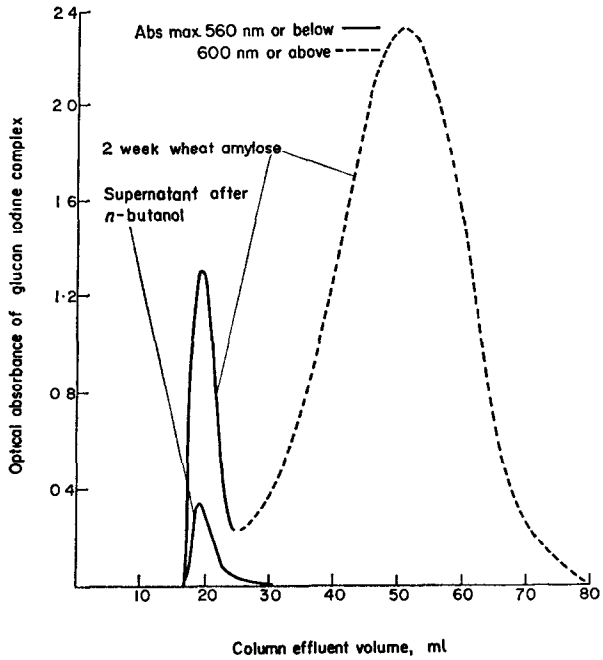

FIG. 3. CHROMATOGRAPHY OF TWO WEEK WHEAT AMYLOSE FROM *n*-BUTANOL FRACTIONATION.

the whole starches and dissolved in dilute sodium chloride. When 2 week wheat amylose was then chromatographed on 2% agarose gel, an excluded fraction with a glucan-iodine absorption maximum at 560 nm was present as well as amylose (Fig. 3). The amylose from 6 week wheat starch also gave an excluded peak but it was smaller. Even potato amylose showed a very small amylopectin peak. *n*-Butanol was then added to the 2 week wheat amylose solution and after centrifuging the amylose-butanol complex, the supernatant was chromatographed. The elution pattern obtained is labelled in Fig. 3 as 'supernatant after *n*-butanol'. The small area of this peak relative to the excluded peak before *n*-butanol treatment indicates that on re-dispersing and complexing for a third time, most of the remaining amylopectin precipitates with the amylose. This may be due to a structural difference in the soluble amylopectin or it may involve association effects with the precipitated amylose.

It is concluded, that as wheat grains and probably other tissues develop, the increase in isopotential binding of the whole starch is due to an increase in the amount of essentially unbranched amylose and is not due to major changes in the structure of this lower molecular weight component, although fractionation by *n*-butanol complexing of immature starches would indicate the latter.

EXPERIMENTAL

Plant material. Wheat (variety Gamut) was marked at anthesis and harvested 14 and 42 days later.

Isolation of starch. Immediately after harvesting, the wheat heads were macerated in 1% aq. NaCl containing 0·01% $HgCl_2$, and toluene (5% of aq.vol.). The macerate was filtered through a double layer of muslin. The residue was macerated and filtered another three times. The combined filtrates were centrifuged (2000 *g* 20 min) and the toluene and aqueous layers discarded. The residue was shaken with the NaCl–toluene solution, passed through double layers of muslin until free of fibrous material and then allowed to settle under gravity. The precipitate was shaken with a water–toluene mixture until no more lipid material separated at the interface. The starch was washed successively with water, ethanol, acetone and ether and dried in a vacuum.

Estimation of nitrogen and phosphorus content. After digestion of the starch with perchloric acid. Nitrogen was estimated with the phenol/nitroprusside method,[16] and phosphorus with a modified Fiske and Subbarow reagent.[17]

Dispersion of starches and fractionation with n-*butanol.* Starch (2 g) was suspended in dimethyl sulphoxide (100 ml) in a bath at 35° and a stream of dry N_2 passed at a constant rate through the solution for 72 hr. The mixture was shaken by hand occasionally in the first 2 hr to avoid the formation of clumps The solution was then poured, with stirring, into ethanol, centrifuged and the precipitate washed successivly by centrifuging with ethanol, analytical grade acetone and peroxide-free ether. It was then suspended in peroxide-free ether. NaCl (0·05 M, 20 ml), free of gaseous oxygen, was added and the starch mixed to a paste. More solvent (180 ml) was added and N_2 (O_2-free) bubbled through the mixture. It was then heated at 100° for 30 min. One-half of the solution was cooled to room temp. and 10% NaN_3 (0·2 ml) added. The other half was cooled to 60° and *n*-BuOH (5 ml) added and mixed. After 48 hr, this half of the solution was centrifuged (14,000 *g*, 30 min at 25°). The supernatant gave the amylopectin fraction. The precipitate was suspended in 0·05 M NaCl (30 ml) (O_2-free) and heated at 100° for 10 min, cooled to 60° and *n*-BuOH (0 15 ml) added. After 48 hr the precipitate was centrifuged (14,000 *g* 30 min at 25°) and dried at room temp. at 0 01 mm pressure. This gave the fraction called 'amylose precipitated as the *n*-butanol complex'. The supernatant gave the mother liquor fraction.

Measurement of isopotential iodine binding of starches and starch fractions. Starch (100 mg) or amylose (40 mg) was wetted with dimethyl sulphoxide (2 ml) in a 50 ml volumetric flask and stood for 18–24 hr. The gel was heated at 100° for 5 min and 0·05 M KI-0·05 M KCl solution added to the calibration mark. An aliquot (20 ml) was transferred by pipette to a 50 ml enclosed titration vessel fitted with a stirrer, platinum electrode and a calomel half cell. The solution was titrated with 0·005 M iodine in 0 05 M KI-KCl, reading the potential 3 min after an addition (accuracy 0·1 mV). Free iodine was determined from a calibration curve without starch. Bound iodine was plotted against free iodine and the 'plateau' region extrapolated to the bound iodine axis.

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Separation of amylose fraction by preparative ultra-centrifugation. A starch solution (2%) was prepared by the previously described procedure. An aliquot (0·2 ml) was applied to a 5·0 ml centrifuge tube, using a band forming cap in an SW65 head. The tube contained a sucrose gradient from 0% to 40% and 0·1 M NaCl, 0·005 M EDTA and 0·02% sodium azide. The tubes were centrifuged at 65,000 rev/min for 180 min, the base pierced and 20 fractions collected. Iodine–KI solution (0·01 M–2 ml) was added to each tube and mixed. 2 ml was diluted to 3·5 ml in a 1-cm spectrophotometer cell and the absorption read at 540–650 nm.

Chromatography of starches and fractions on agarose gel. A 1% solution of glucan (1 ml) was applied to a column of 2% agarose gel (Pharmacia Sepharose 2B, exclusion limit for polysaccharides 20×10^6) at 25·0°. The column was eluted with a solution containing 0·1 M NaCl, 0·005 M EDTA and 0·02% sodium azide, and about 35 fractions collected. An aliquot of each fraction was mixed with 0·02% iodine in KI solution and the absorption read at 560 to 650 nm.

Determination of glucan-iodine absorption spectra and α(1–4) (1–6) *glucan contents of retarded (amylose) fractions.* An aliquot (15 ml) of the retarded fraction was mixed with 0·2 M acetate buffer (pH 4·5) and amyloglucosidase solution (0·5 ml) (containing 2 mg/ml of Sigma amyloglucosidase) at 40° for 30 min. Aliquots (5 ml) were then taken for estimation of reducing sugar by the Nelson-Somogyi method. The glucan–iodine absorption spectra were measured by adding 0·02% iodine–KI solution (2 ml) to an aliquot of the retarded fraction (1·2 ml). Potato solutions were diluted to twice the total volume with iodine solution and water before reading.

Large scale fractionation of 2 week starch by agarose gel chromatography. Starch solution (1%) was applied in 20 ml portions to a column of 2% agarose gel (55·0 × 5·5 cm). Fractions (20–25 ml) were collected and 1 ml aliquots taken to measure the glucan–iodine absorption spectra. Five portions were chromatographed. Fractions with an absorption maximum at 600 nm or above (3500 ml) were combined. This solution was concentrated to a volume of 50 ml in a membrane filtration cell (Diaflo model 50), using a membrane with a nominal molecular weight cut-off point of 10,000 (Diaflo UM-10 membrane) and a positive pressure of 45 lb/in^2 *n*-Butanol (2·5 ml) was added. After 48 hr the precipitate was collected by centrifugation (14,000 *g*, 30 min) and dried at room temperature at 0·01 mm pressure.

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